Kinetics of $^{13}$N-ammonia uptake in myocardial single cells indicating potential limitations in its applicability as a marker of myocardial blood flow

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ABSTRACT To study kinetics and principles of cellular uptake of $^{13}$N-ammonia, a marker of coronary perfusion in myocardial scintigraphy, heart muscle cells of adult rats were isolated by perfusion with collagenase and hyaluronidase. Net uptake of $^{13}$N, measured by flow dialysis, reached equilibrium within 20 sec in the presence of sodium bicarbonate and carbon dioxide (pH 7.4, 37°C). Total extraction, 80 sec after the reaction start, was 786 ± 159 μmol/ml cell volume. Cells destroyed by calcium overload were unable to extract $^{13}$N-ammonia. Omission of bicarbonate and carbon dioxide reduced total extraction to 36% of control. $^{13}$N-Ammonia uptake could also be reduced by 50 μM 4,4′-disothiocyanostilbene 2,2′ disulfonic acid, by 100 μg/ml 1-methionine sulfoximine, and by preincubation with 5 μM free oleic acid. These results indicate that in addition to metabolic trapping by glutamine synthetase, the extraction of $^{13}$N-ammonia by myocardial cells is influenced by cell membrane integrity, intracellular-extracellular pH gradient, and possibly an anion exchange system for bicarbonate. For this reason, the uptake of $^{13}$N-ammonia may not always provide a valid measurement of myocardial perfusion.


$^{13}$N-ammonia ($^{13}$N),* a positron-emitting radionuclide, has been used as a marker of myocardial perfusion.1-8 Because cellular extraction of an ideal flow marker must be proportional to regional myocardial blood flow, extraction of such a marker should not be influenced by cell metabolism or cell membrane transport mechanisms. Since myocardial extraction fraction of $^{13}$N-ammonia can be significantly reduced when intracellular glutamine synthetase is inhibited,6,7 the usefulness of this compound as a flow marker has been questioned. This study provides additional data regarding the kinetics and possible role of the plasma membrane in myocardial $^{13}$N uptake and retention in isolated adult rat heart cells.

Materials and methods

Preparation of myocardial single cells. Male Wistar rats 3 to 4 months old were heparinized and anesthetized by urethane (1.5 mg/g body weight, intraperitoneally). Hearts were extracted and perfused in a nonrecycling manner for 10 min with 35 mM NaCl, 4.75 mM KCl, 1.2 mM KH₂PO₄, 150 mM sucrose, 25 mM NaHCO₃, 10 mM HEPES buffer, 10 mM glucose, and 5 g/liter albumin bovine fraction V (pH 7.4, 37°C, 95% O₂/5% CO₂).9 A recycling perfusion (25 min) was carried out with the same solution also containing 150 U/ml unpurified collagenase (E.C.3.4.1.19.) and 1000 U/ml hyaluronidase (E.C.3.2.1.36.). At the end of the perfusion the softened heart was sliced into small pieces and reinculated for 10 min in the same enzyme-containing solution; the suspension was filtered through a nylon mesh and centrifuged gently for 2 min at 50 g. The supernatant was removed and the pellet was washed three times in 130 mM NaCl, 4.0 mM KCl, 1.2 mM KH₂PO₄, 25 mM HEPES buffer, 10 mM glucose, and 20 g/liter albumin bovine fraction V (pH 7.4, room temperature, 100% O₂). A small population of completely destroyed cells in these cell suspensions could be easily distinguished from cells with a morphologically intact structure by light microscopy. Intact cells were typically rod shaped, cross-striated, and sometimes showed spontaneous irregular contractions. A stepwise return to physiologic concentrations of calcium was possible after the cells were suspended for 15 min in 100 mM KCl, 30 mM KH₂PO₄, 1.2 mM MgSO₄, 20 mM taurine, 5.5 mM ATP, 3.0 mM pyruvate, 5.0 mM succinate,

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5.0 mM β-hydroxybutyrate, 10 mM glucose, 0.05 mM EGTA, and 5 g/liter albumin bovine fraction V (pH 7.4, 4°C). The sedimented cells were resuspended four times in the reaction medium described below, containing increasing concentrations of total calcium (0.1, 0.5, 2.0, and 3.5 mM). These cell suspensions contained about 65% to 80% intact cells that did not contract spontaneously; however, rhythmic contractions could be induced by the external application of current impulses. Electron micrographs revealed alterations of the glycocalyx as described by Isenberg et al., but other ultrastructural features were not abnormal.

Nonviable cells were prepared by incubation of cells in a reaction medium containing 3.5 mM CaCl₂ immediately after perfusion with collagenase and hyaluronidase. The separated cells prepared in this manner showed the typical “calcium paradox,” i.e., they were destroyed by calcium overload.

Preparation of electron micrographs. Isolated heart muscle cells were fixed in 2.5% glutaraldehyde containing 0.3 M Sorensen’s buffer, after which the cells were centrifuged at 1000 rpm for 10 min, postfixed in 1% OsO₄, dehydrated in ethanol, and embedded in epon-araldite. Ultrathin sections were stained with lead citrate and uranylacetate and examined with a Zeiss EM 10 microscope.

Reaction media. Two different reaction media, medium I and medium II, were used in the measurements of [¹³N] uptake. Medium I contained 110 mM NaCl, 4.75 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 20 mM HEPES buffer, 25 mM NaHCO₃, 10 mM glucose, 5 g/liter fatty acid-free bovine albumin, and 3.5 mM CaCl₂. This medium was equilibrated by 5% CO₂ and 95% O₂ at pH 7.4. Medium II, gassed with 100% O₂, had all the components of medium I except NaHCO₃, and the NaCl concentration was 135 mM (pH 7.4).

For intervention studies the suspension, containing calcium tolerant cells, was diluted to 1.5 ml, slowly mixed, and divided into two equal parts. Both parts were gently centrifuged (2 min at 50 g) and the supernatants were discarded. One pellet was always resuspended in 0.75 ml of medium I (control); the other was resuspended in 0.75 ml medium II or in medium I containing either 50 µM 4,4'-dithiobis(3-nitrobenzoic) acid (DNB) or 100 µM 4,4'-diaminodiphenylsulfone (DNDS) as described above, the freely diffusible part of ammonium chloride in the reaction chamber; C₂ = concentration of [¹³N]-ammonium chloride in the flow chamber; V = volume of the flow chamber; f = flow rate of the reaction medium (see text for details).

FIGURE 1. Schematic description of the experimental set-up, in which the reaction chamber is separated from the flow chamber by a dialysis membrane. The gamma counter automatically recorded the total counts of the effluent at 5 sec intervals. The concentration of [¹³N] in the flow chamber (C₂) reached an equilibrium at a time (t) that depended on flow rate (f = 12.8 ml/min., total volume of the flow chamber plus that of the tube leading to the gamma counter (V = 0.90 ml), the membrane constant D, and the concentration of the diffusible part of [¹³N]-ammonium chloride in the reaction chamber (C₁) according to the following equation:

\[ C₂ = C₁D(f - e^{-\frac{f}{V}}) \]

The decrease of the total amount of [¹³N] in the reaction chamber caused by diffusion into the flow chamber during the experiment was below 0.5% and could be neglected. To get a standard count rate per mol [¹³N], the count rates of three aliquots of the reaction suspension corrected for physical decay were measured after each experiment and related to the total amount of [¹³N] in the reaction chamber.

From the above equation, the membrane constant D was obtained by duplicate calibration measurements carried out without cells in the reaction medium. Figure 2 gives an example of the time course of [¹³N] activity in the flow chamber, corrected for physical decay. Because 20 sec were required before equilibrium was reached in the flow chamber, no interventions were made during the uptake reaction. To estimate the amount of extracted [¹³N] at 5, 10, and 15 sec, the corresponding apparent membrane constants, as obtained from the calibration curves, were used. The apparent initial rate of [¹³N] uptake was determined from the slope given by the 5 and 10 sec values.

After determination of D and calculation of the count rate per mole [¹³N] as described above, the freely diffusible part of [¹³N] in the reaction chamber was calculated from [¹³N] activity measured in the flow chamber. From the difference between the amount of freely diffusible [¹³N] in the absence and presence of cells in the reaction medium, the amount of [¹³N] extracted by myocardial cells was calculated for each time interval, and then related to total cell volume that was estimated by calculation of (⁴H)-water and (¹⁴C)-inulin space in the cell suspension of the control and intervention phases as described by Powell et al. [¹⁴H] and [¹⁴C] were measured in a liquid scintillation counter (Tri-Carb 4500; Packard Instruments, Frankfurt, F.R.G.), with count rates corrected for background and channel overlap.
Materials. 13N-Ammonia was produced in a cyclotron, according to the 16O(P,α)13N reaction.15 The following materials were obtained from Sigma Chemie GmbH, Taufkirchen, F.R.G.: disodium salt of DIDS, 1-methionine sulfoximine, bovine serum albumin fraction V, sodium salt of oleic acid, and fatty acid-free bovine serum albumin (fraction V). Hyaluronidase was obtained from Serva Feinbiochemica, Heidelberg, and collagenase type CLS I No. 495245P was purchased from Seromed GmbH, Munchen, F.R.G.

Myocardial cells were stimulated by a SD-SC stimulator (Grass Medical Instruments, Quincy, MA). Dialysis membranes were purchased from Kalle, Hoechst, F.R.G. (pore diameter 2.5 to 8.0 nm).

To measure 13N activity, a gamma counter from Berthold, Wildbad, F.R.G. (H.V. supply BF 2302, ratemeter LB 2232, scaler timer BF 2270-1, interface BF 2222 TEL) and a NaI(Tl) crystal were used (type 7SF8/G; Harshaw Chemie B.V., The Netherlands).

Results

All data obtained in this study are summarized in table 1. As described in Materials and methods, cell preparations were contaminated by irreversibly destroyed cells (table 1), which could not be removed without significantly reducing the total amount of intact myocardial cells. For this reason the role of unspecific binding of 13N by nonviable cells was determined. There was no difference between the calibration curve without cells and the curve obtained by suspending destroyed cells in the reaction medium (figure 2, left). Dead cells therefore did not extract measurable amounts of 13N.

The cellular extraction of 13N by myocytes under control conditions (medium I, 95% O2, 5% CO2, pH 7.4, 37° C) was rapid, reaching an equilibrium within 15 sec (figure 3). The apparent initial rate of 13N uptake was reduced to 9% of the control value when cells were suspended in medium II, in which sodium bicarbonate was replaced by HEPES buffer and carbon dioxide was omitted. Total uptake, 80 sec after the reaction started, was 36% of the control value.

Since the dependence of 13N uptake on the presence of bicarbonate and carbon dioxide could be explained if cellular 13N uptake were mediated by an anion exchange system, the effect of DIDS was investigated. As shown in figure 4, 50 μM DIDS depressed total uptake.

![FIGURE 2. Time course of 13N activity in the flow chamber, corrected for physical decay. Left, Calibration curve without cells in the reaction medium (C) and a similar curve obtained when destroyed cells were present in the reaction medium (X) (standard count rate corrected for physical decay = 6.12 x 106 cpm/μmol 13N; D = 4.4 x 10^-4 ml/min; 37° C; pH 7.4; result of one of five replicate experiments). Right, Calibration curve of another experiment without cells in the reaction medium (C) (standard count rate corrected for physical decay = 7.51 x 106 cpm/μmol 13N; D = 4.45 x 10^-4 ml/min; 37° C; pH 7.4) together with the curve obtained when intact cells (33 μl/500 μl) were present in the reaction medium (●). Total cellular 13N extraction was calculated from the difference of the amount of 13N activity in the flow chamber as described in the text.](http://circ.ahajournals.org/content/71/1/389)

![TABLE 1 13N uptake in myocardial single cells](http://circ.ahajournals.org/content/71/1/389)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Apparent initial rate of 13N uptake (pmol/cm²sec)</th>
<th>Total 13N uptake (nmol/ml)</th>
<th>Fraction of nonviable cells (%)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140 ± 55</td>
<td>786 ± 159</td>
<td>25.1 ± 7.4 (26.9 ± 7.0)</td>
<td>21</td>
</tr>
<tr>
<td>Medium II</td>
<td>12 ± 8</td>
<td>285 ± 80</td>
<td>25.0 ± 5.9 (26.7 ± 6.6)</td>
<td>6</td>
</tr>
<tr>
<td>Medium I + 50 μM DIDS</td>
<td>37 ± 28</td>
<td>280 ± 60</td>
<td>27.3 ± 7.5 (27.6 ± 8.1)</td>
<td>6</td>
</tr>
<tr>
<td>Medium I + 100 μM DIDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-methionine sulfoximine</td>
<td>48 ± 29</td>
<td>330 ± 110</td>
<td>20.2 ± 5.7 (22.5 ± 5.3)</td>
<td>4</td>
</tr>
<tr>
<td>Medium I + 5 μM oleic acid</td>
<td>18 ± 14</td>
<td>180 ± 88</td>
<td>27.8 ± 5.3 (29.8 ± 6.9)</td>
<td>5</td>
</tr>
</tbody>
</table>

AFor calculation of the apparent initial rate, a surface area of 530 cm²/g myocardium was assumed.16, 17
BFractions of nonviable cells before and after the observation period (in parentheses) in percent of the total cell amount.
CVValues are mean ± SD.
uptake to 35% of the control value. A similar effect was seen in 50 μM 4,4′dinitrostilbene2,2′disulfonylic acid (data not shown).

Low concentrations of oleic acid (figure 5) reduced the apparent initial rate to 13% and total uptake to 23% of the control value. As demonstrated by Bergmann et al. and Schelbert et al., who measured 13N uptake and washout in the whole heart of rabbits and dogs, respectively, 13N uptake could be depressed by l-methionine sulfoximine, an inhibitor of intracellular glutamine synthetase. In the presence of 100 μg/ml l-methionine sulfoximine, apparent initial rate and total uptake were reduced to 34% and 42% of the control values, respectively (figure 6). The cells remained morphologically intact in the presence of DIDS, oleic acid, and l-methionine sulfoximine, and both tolerated extracellular calcium and responded with ordered contractions to electrical stimuli.

Discussion

13N-Ammonia has been used as a flow marker in myocardial scintigraphy, but the ability of this tracer to quantify regional myocardial blood flow has been questioned because 13N uptake and retention depend on metabolic trapping as well as on myocardial perfusion. The results of this study indicate that cellular uptake of 13N also depends on the integrity of the plasmalemmal membrane and possibly on an anion exchange system that can be inhibited by stilbene disulfonic acids.

Single heart muscle cells prepared from adult rats were used to eliminate influences of regional blood flow, blood vessel wall, and interstitial space. Apart from alterations of the external lamina of the glycosalyx, the cells were morphologically intact as shown by

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**FIGURE 3.** Calculated net uptake of 13N in medium I under control conditions (●) and in medium II when sodium bicarbonate was replaced by HEPES buffer and CO2 was omitted (○). Values are mean ± SD (n = 6).

**FIGURE 4.** Influence of DIDS on cellular 13N uptake in medium I (gassed with 5% CO2 and 95% O2). ● = control; ○ = preincubation with 50 μM DIDS. Values are mean ± SD (n = 6).

**FIGURE 5.** Time course of cellular 13N accumulation in the absence (●) and presence of 5 μM free oleic acid (○). Experiments were carried out with a slightly modified reaction medium (medium I, no albumin), as described in the text. Cells were preincubated in the oleic acid containing medium 1 30 min before the reaction was started. Values are mean ± SD (n = 5).

**FIGURE 6.** Cellular 13N uptake in the absence (●) and presence of 100 μg/ml l-methionine sulfoximine (○). Experimental conditions are described in Materials and methods. Values are mean ± SD (n = 4).
electron microscopy. The cells were also calcium tolerant and showed rhythmic contractions only after the application of current impulses. As demonstrated by metabolic and electrophysiologic studies,9,10,22-24 these features provide strong criteria for cell viability, and loss of the external lamina of the glycocalyx does not interfere with major functions of the cell membrane such as the slow inward current,10 electromechanical coupling,10 activation of adenylate cyclase,22 and insulin-stimulated glucose transfer.23 Although dead cells could seriously interfere with the results by binding 13N nonspecifically, our results demonstrated that dead cells did not extract significant amounts of 13N.

The flow dialysis system used in this study allowed the uptake of the short-lived 13N isotope to be measured and largely avoided mechanical alterations of the cells during the experiment. However, the flow dialysis system is suitable only if cellular extraction of the tracer is sufficiently high to reduce markedly the extracellular concentration during the experiment. Because there was a delay of 20 sec until the flow system reached equilibrium (figure 2), the membrane constants calculated from calibration measurements at 5, 10, and 15 sec were only apparent, so that the calculations based on these apparent membrane constants can give only approximations of the real uptake velocity.

The calculated amount of 13N extracted by myocardial cells does not represent the concentration of ammonium chloride in the cytosol because the amount of intracellular ammonium chloride present before the start of the reaction was not taken into account in this study. Furthermore, a significant amount of the accumulated 13N was metabolized by the glutamine synthetase pathway (figure 7, step 7), as evidenced by the marked reduction of total 13N uptake by 1-methionine sulfoximine, a known glutamine synthetase inhibitor.18-21 A similar reduction of 13N uptake by 1-methionine sulfoximine has been observed in perfused rabbit and dog hearts. Additional pathways, such as the urea cycle, could also metabolize 13N.25

The uptake and retention of 13N could also be influenced by changes in the translocation through the sarcolemma since 13N participates in an acid-base equilibrium between ammonium ions and NH3. The ammonium ion is unlikely to cross the lipid bilayer by simple diffusion,26 but this ion can serve as a potassium analog in the activation of the sarcolemmal Na-K-ATPase.27,28 Since extracellular ammonium concentrations in the present study were well below the Km of the complex of ammonium with the Na-K-ATPase measured in other tissues, it is unlikely that the amount of 13N in the reaction medium activated this ion transport system. Although specific membrane potassium channels could also participate in 13N translocation, studies of the ion selectivity of potassium channels showed a permeability ratio between ammonium and potassium ions of only 0.13.29

Lipid-soluble NH3, however, is known to cross the lipid bilayer rapidly30 (figure 7, step 2), and the association of protons with intracellular NH3 is also very rapid31 (figure 7, step 1). Because plasmalemmal permeability to NH4+ is lower than that to NH3, the distribution of NH4+ across the sarcolemma will be determined largely by the intracellular-extracellular pH gradient, as shown in studies of the distribution of ammonia between blood and cerebrospinal fluid.32

Intracellular pH has been reported to be 0.2 to 0.5 units lower than extracellular pH.33-35 This would result in an intracellular-extracellular concentration ratio of NH4+ of 1.6 to 3.2, provided intracellular alkalization induced by NH3 influx36,37 is compensated for by a buffer system. Intracellular pH could be maintained by an anion exchange system as it has been described in red blood cells and in sheep cardiac Purkinje fibers38-45 (figure 7, step 6). The ability of stilbene disulfonic acids and the absence of carbon dioxide and bicarbonate to inhibit 13N uptake suggests that an anion exchange system is present in the sarcolemma of rat myocytes. Stilbene disulfonic acids such as DIDS and DNDS are known to be specific inhibitors of anion exchange systems in erythrocytes.40,41 Carbon dioxide plays an important role in regulating intracellular pH, so that if CO2 rapidly crosses the plasma membrane46 and is metabolized within the cell to H+ and HCO3- via the carbonic anhydrase reaction (figure 7, step 5), the reduction of intracellular buffer capacity in the absence of CO2 could decrease cellular 13N uptake. Dependence of ammonia uptake on CO2 has been demonstrated earlier in skeletal muscle and in erythro-

FIGURE 7. Proposed reaction scheme of 13N uptake into the myocardial cell. GS = glutamine synthetase; Glu = glutamate; Gln = glutamin; i = intracellular space; o = extracellular space; CA = carbonic anhydrase. See text for details.
Because the intracellular-extracellular pH gradient has been reported to be reduced in hypercapnic acidosis,\(^ {51, 52}\) the moderate reduction of \(^ {13}\)N extraction when plasma pH is lowered (Schelbert et al.\(^ {4}\)) may reflect the putative participation of the intracellular-extracellular pH gradient in the regulation of \(^ {13}\)N uptake.

A number of studies suggest that changes in lipid metabolism lead to altered cardiac function,\(^ {53, 54}\) and free fatty acids have been shown to modify the Na-K-ATPase,\(^ {54}\) the calcium pump ATPase of sarcoplasmic reticulum,\(^ {55}\) and cell membrane fragility.\(^ {56}\) This study extends these observations with the finding that oleic acid reduces \(^ {13}\)N uptake. Since the oleic acid concentrations used in this study neither caused morphologic alterations of the cells nor changed extracellular pH, the reduction of \(^ {13}\)N uptake could be explained by a functional change in the cell membranes such as inhibition of the postulated anion exchange system or a change in membrane permeability leading to an increase in \(^ {13}\)N efflux. However, further studies are needed to evaluate these and other possible mechanisms of this effect of oleic acid.

The data presented in this report indicate that \(^ {13}\)N-ammonia may not be a valid marker for regional myocardial blood flow under all conditions because \(^ {13}\)N-ammonia uptake depends not only on perfusion but also on the viability of the myocardial cells, metabolic factors, and the function of the plasma membrane. Since ammonia is metabolized by glutamine synthetase in a reaction that requires ATP, the extent of ammonia metabolism may depend on the energetic state of the myocardial cell. Thus the reduction of intracellular ATP to concentrations in the range of the \(K_m\) for the enzyme-ATP complex, which is about 1.5 mM in rat skeletal muscle,\(^ {57}\) could reduce intracellular \(^ {13}\)N metabolism by glutamine synthetase and therefore inhibit \(^ {13}\)N extraction. As demonstrated by the effect of oleic acid, changes in lipid metabolism could also influence the capability of the myocardial cell to accumulate \(^ {13}\)N-ammonia. Furthermore, changes of the intracellular-extracellular pH gradient caused by intracellular and extracellular acidosis during ischemia\(^ {53}\) or by dysfunction of the postulated anion exchange system could influence \(^ {13}\)N uptake. These factors may complicate the interpretation of clinical myocardial blood flow measurements by \(^ {13}\)N-ammonia scintigraphy under different pathologic conditions.

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